RESEARCH NOTE

REVISED A nonlinear and time-dependent leak current in the presence of calcium fluoride patch-clamp seal enhancer [version 2; peer review: 3 approved, 1 approved with reservations]

Chon Lok Lei[1-3], Alan Fabbri[4], Dominic G. Whittaker[5], Michael Clerx[3], Monique J. Windley[6,7], Adam P. Hill[6,7], Gary R. Mirams[5*], Teun P. de Boer[4*]

[1]Institute of Translational Medicine, Faculty of Health Sciences, University of Macau, Macau, China
[2]Department of Biomedical Sciences, Faculty of Health Sciences, University of Macau, Macau, China
[3]Department of Computer Science, University of Oxford, Oxford, Oxfordshire, OX1 3QD, UK
[4]Department of Medical Physiology, Division of Heart and Lungs, University Medical Centre Utrecht, Utrecht, 3584 CX, The Netherlands
[5]Centre for Mathematical Medicine & Biology, School of Mathematical Sciences, University of Nottingham, Nottingham, Nottinghamshire, NG7 2RD, UK
[6]Molecular Cardiology & Biophysics Division, Victor Chang Cardiac Research Institute, Darlinghurst, New South Wales, 2010, Australia
[7]St Vincent's Clinical School, UNSW Sydney, Darlinghurst, New South Wales, 2010, Australia

[*] Equal contributors

v2 **First published:** 01 Jul 2020, **5**:152
https://doi.org/10.12688/wellcomeopenres.15968.1
Latest published: 02 Nov 2021, **5**:152
https://doi.org/10.12688/wellcomeopenres.15968.2

Abstract

Automated patch-clamp platforms are widely used and vital tools in both academia and industry to enable high-throughput studies such as drug screening. A leak current to ground occurs whenever the seal between a pipette and cell (or internal solution and cell in high-throughput machines) is not perfectly insulated from the bath (extracellular) solution. Over 1 GΩ seal resistance between pipette and bath solutions is commonly used as a quality standard for manual patch work. With automated platforms it can be difficult to obtain such a high seal resistance between the intra- and extra-cellular solutions. One suggested method to alleviate this problem is using an F^- containing internal solution together with a Ca^{2+} containing external solution — so that a CaF_2 crystal forms when the two solutions meet which 'plugs the holes' to enhance the seal resistance. However, we observed an unexpected nonlinear-in-voltage and time-dependent current using these solutions on an automated patch-clamp platform. We performed manual patch-clamp experiments with the automated patch-clamp solutions, but no biological cell, and observed the same nonlinear time-dependent leak current. The

Open Peer Review

Reviewer Status ✓ ? ✓ ✓

	Invited Reviewers			
	1	**2**	**3**	**4**
version 2 (revision) 02 Nov 2021	✓ report		✓ report	✓ report
version 1 01 Jul 2020	? report	? report	? report	? report

1. **Gildas Loussouarn**, University of Nantes, Nantes, France

2. **Andrew Glazer**, Vanderbilt University Medical Center, Nashville, USA

current could be completely removed by washing out F^- ions to leave a conventional leak current that was linear and not time-dependent. We therefore conclude fluoride ions interacting with the CaF_2 crystal are the origin of the nonlinear time-dependent leak current. The consequences of such a nonlinear and time-dependent leak current polluting measurements should be considered carefully if it cannot be isolated and subtracted.

Keywords
electrophysiology, leak current, automated patch, patch clamp, seal enhancer

3. **Marc Rogers**, Metrion Biosciences Limited, Cambridge, UK

4. **Carlos Guillermo Vanoye**, Northwestern University, Chicago, USA

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Chon Lok Lei (chonloklei@um.edu.mo)

Author roles: Lei CL: Conceptualization, Investigation, Methodology, Software, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Fabbri A**: Investigation, Writing – Review & Editing; **Whittaker DG**: Investigation, Methodology, Writing – Review & Editing; **Clerx M**: Methodology, Software, Supervision, Writing – Review & Editing; **Windley MJ**: Investigation, Writing – Review & Editing; **Hill AP**: Conceptualization, Funding Acquisition, Supervision, Writing – Review & Editing; **Mirams GR**: Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Project Administration, Supervision, Writing – Review & Editing; **de Boer TP**: Conceptualization, Funding Acquisition, Investigation, Methodology, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Wellcome Trust through a Senior Research Fellowship to GRM [212203]. This work was also supported by the Engineering and Physical Sciences Research Council and the Medical Research Council [EP/L016044/1]; the Biotechnology and Biological Sciences Research Council [BB/P010008/1]; the Australian Research Council [DP190101758]; and the ZonMW MKMD programme [114021501 and 114022502]. CLL acknowledges support from the Clarendon Scholarship Fund; and the EPSRC, MRC and F. HoffmannLa Roche Ltd. for studentship support. GRM and DGW acknowledge support from the Wellcome Trust via a Wellcome Trust Senior Research Fellowship to GRM. MC acknowledges support from a BBSRC project grant. APH and MJW acknowledge support from Victor Chang Cardiovascular Innovation Centre and ARC. AF and TPdB acknowledge support from the ZonMW MKMD programme.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Lei CL, Fabbri A, Whittaker DG *et al.* **A nonlinear and time-dependent leak current in the presence of calcium fluoride patch-clamp seal enhancer [version 2; peer review: 3 approved, 1 approved with reservations]** Wellcome Open Research 2021, **5**:152 https://doi.org/10.12688/wellcomeopenres.15968.2

First published: 01 Jul 2020, **5**:152 https://doi.org/10.12688/wellcomeopenres.15968.1

REVISED **Amendments from Version 1**

- There are many changes to the text to reflect that this nonlinear leak in the presence of calcium fluoride is not unique to automated patch but a general problem in any patch-clamp platform.
- New experimental recordings were undertaken with Sylguard to increase the replicates of the experiment, these can be seen in Figure 4.
- Figure 2, Figure 3 and Figure 4 now present summary curves for the current-voltage relationship and time constants derived from multiple recordings.
- A new Figure 3 illustrates how time constants and steady state currents were derived from the recordings and gives the time constants across the different experimental settings.
- All of the new datasets, analysis code, and code to produce figures are available in the updated code repository - see Data Availability.

Any further responses from the reviewers can be found at the end of the article

Introduction

Voltage-clamp and current-clamp configurations of the patch clamp technique have been vital tools for studying electrophysiology since the time of Hodgkin & Huxley (1952). Voltage-clamp experiments are commonly used to study voltage and time dependence of ion currents, while current-clamp experiments are used to study for example action potentials of excitable cells. Many different techniques have been developed and one of most the widely-used methods is whole-cell patch clamping (Sakmann & Neher, 1984).

Whole-cell patch-clamp experiments can be performed using either manual control of a pipette's position or on automated high-throughput machines based on pores and microfluidics. Manual patch is the conventional method, but it can be very time consuming and low-throughput; whilst automated platforms allow high-throughput recordings, which can be extremely useful for studies that require high numbers of measurements such as drug screening in the pharmaceutical industry (Bell & Fermini, 2021; Elkins *et al.*, 2013). In recent years many studies have begun to use automated patch-clamp systems to study ion channel electrophysiology (Gertler *et al.*, 2019; Kang *et al.*, 2019; Kozek *et al.*, 2020; Lei *et al.*, 2017; Lei *et al.*, 2019a; Lei *et al.*, 2019b; Li *et al.*, 2017; Ng *et al.*, 2020; Toh *et al.*, 2020; Vanoye *et al.*, 2018).

A schematic comparison of the two patch-clamp methods is shown in Figure 1A–B. Manual patch-clamp uses a fire-polished glass pipette to form a tight electrical seal (~GΩ) between the pipette tip and the cell membrane. Although the composition of the ionic solutions in the pipette and bath depends on the type of experiments, these solutions are usually intended to be similar to the relevant physiological conditions. Automated platforms, on the other hand, usually have a very different configuration to manual patch, see Figure 1B; they use a design where the cells are suspended on top of a micro-pore on a planar surface. However, this planar design does not always yield as tight a seal (~hundreds of MΩ) as the conventional manual patch-clamp. As a result, some systems recommend the use of seal enhancers that rely on the presence of certain additional ions in the two solutions. For instance, a F^- containing internal solution together with a Ca^{2+} containing external solution has been used with in both early planar microfluidics setups (Kostyuk *et al.*, 1975) as well as manual patching (Maltsev & Undrovinas, 1997; Rugiero *et al.*, 2003; Volkers *et al.*, 2013; Wang *et al.*, 1996; Wendt *et al.*, 1992), and is now employed in many automated platforms (including over 80% of the reported solutions in a recent cross-site and cross-platform comparison of drug screening for a panel of cardiac ion channels, Kramer *et al.*, 2020). The improvement of seal resistance in the presence of these solutions is thought to be due to the formation of CaF_2 crystals at the interface between the pipette or micro-pore and the cell (Løjkner *et al.*, 2019), as illustrated schematically in Figure 1B.

Studies have compared manual patch clamping with automated patch clamping data, and showed that their performances are similar (Billet *et al.*, 2017). Here we examine the reason for some unusual kinetics (dynamics) of a leak current that we observed first on an automated platform, before finding it also appeared in manual patch clamp experiments in the presence of CaF_2.

Figure 2A shows an automated patch (Nanion SyncroPatch 384PE) recording of the leftover current measured on Chinese hamster ovary (CHO) cells transfected with the human Ether-à-go-go-Related Gene (hERG)1a after applying a hERG-specific blocker (in this case 0.5 μM of E-4031) at 25°C, with capacitance and 80% series resistance compensations according to manufacturer's settings. The measurements were done under a voltage-clamp protocol used in Lei *et al.* (2019a), known as the "staircase protocol" (see top panel of Figure 2). One might assume this remaining current consists of both leak current (due to finite resistance of the seal), leftover incompletely blocked hERG, and/or ion currents conducted by non-hERG ion channels natively present in the CHO cells (which we will refer to as 'endogenous currents'). The measurements (blue) are consistent across laboratories. In Figure 2 we show measurements taken at: (A) F. Hoffmann-La Roche, Basel (Lei *et al.*, 2019a; Lei *et al.*, 2019b); and (B) Victor Chang Cardiac Research Institute in Sydney (using the same type of Nanion SyncroPatch machine).

The leftover currents are time-dependent when the cell is clamped at a constant voltage; their (normalised) current-voltage (I-V) relationships (at approximately steady state, extrapolated from currents during steps, as described later and in Figure 3A) are non-Ohmic (nonlinear), as shown in Figure 2's (right panels) blue boxplots for n cells (n = 15 and n = 10). Therefore we refer them to as "nonlinear time-dependent" currents. Measurements were repeated with non-hERG transfected wild-type CHO cells using a manual patch-clamp system (n = 4) and the same automated patch-clamp platform (n = 32),

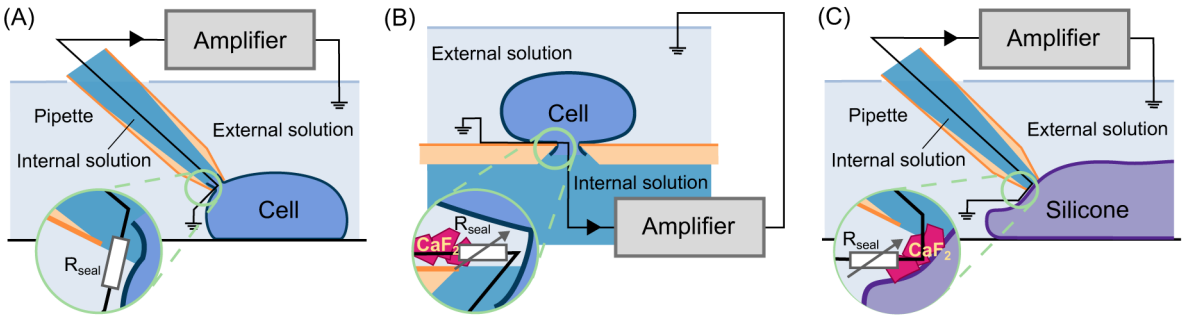

Figure 1. A schematic comparison of manual and automated patch-clamp methods, with a cartoon representation of the leak current circuit. (**A**) Shows the conventional manual patch-clamp, where a polished glass pipette is used to form a tight electrical seal. (**B**) Shows the planar design of an automated patch-clamp, where the cell is suspended on top of a micro-pore in the presence of CaF_2. (**C**) Shows the set-up of our manual patch-clamp silicone experiments with automated patch-clamp solutions. The magnifications show the difference between the leak current from the three configurations.

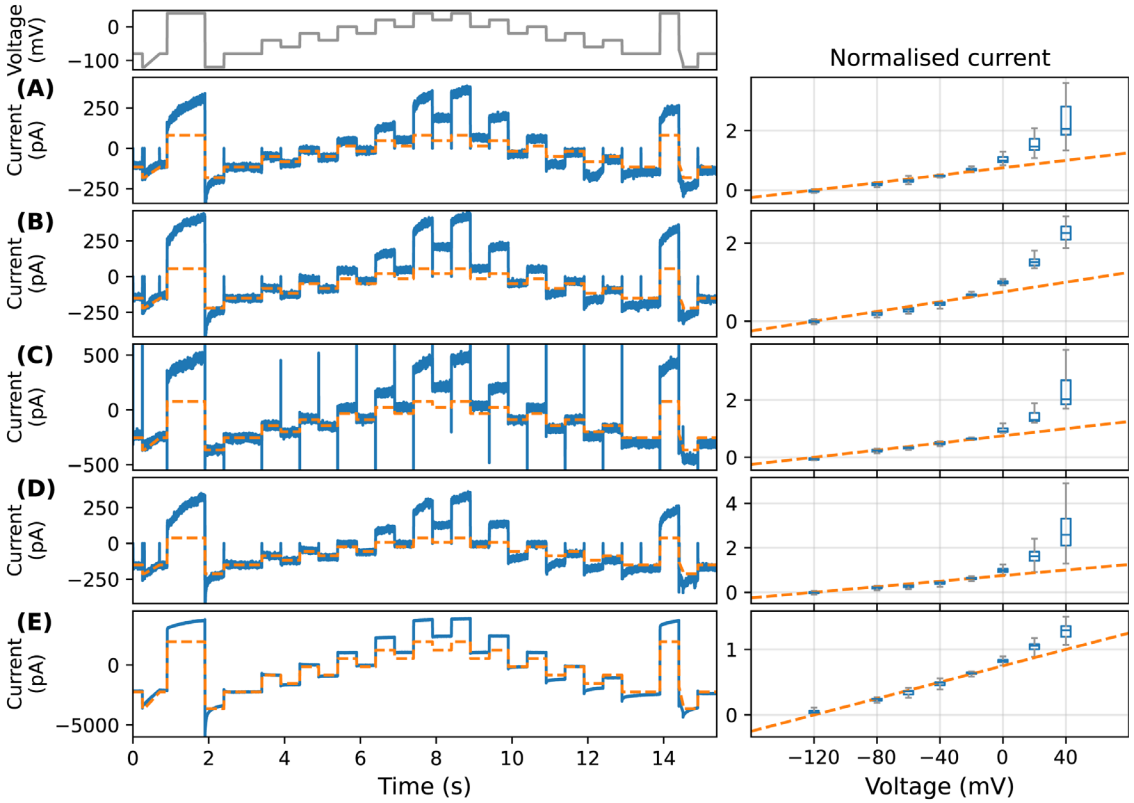

Figure 2. Examples of nonlinear time-dependent leftover current in the presence of CaF_2. On the left shows typical time series recordings under the staircase protocol (top panel) (Lei *et al.*, 2019a); right shows the normalized current-voltage (I-V) relationships (at steady state) with a boxplot (range and quartiles) for technical replicates (n). Note that each boxplot can contain more than one data point per cell due to the repeated voltage steps in the protocol. Experimental recordings are shown in blue, and linear leak estimations are shown in dashed orange. The I-V plots are normalized such that the minimum fitted linear leak current (value of the orange line) is 0 and the maximum is 1; note therefore that reversal potential is not at zero on these plots, but this is a simple way to compare results from many cells when leftover current appears to reverse at different voltages. Recordings of the leftover current measured on hERG1a transfected CHO cells after applying an I_{Kr}-specific blocker (0.5 μM of E-4031) are shown, where experiments were performed at (**A**) F. Hoffmann-La Roche, Basel (Lei *et al.*, 2019a; Lei *et al.*, 2019b), n = 15 and (**B**) an independent reproduction at Victor Chang Cardiac Research Institute in Sydney, n = 10, both using a SyncroPatch 384 automated patch-clamp platform. (**C** and **D**): Recordings with non-transfected CHO cells, using (**C**) a manual patch-clamp system (n = 4) and (**D**) the automated patch-clamp platform (n = 32). (**E**) Shows a typical recording for an empty well-plate (no biological cells in the solution) in the automated platform (n = 20).

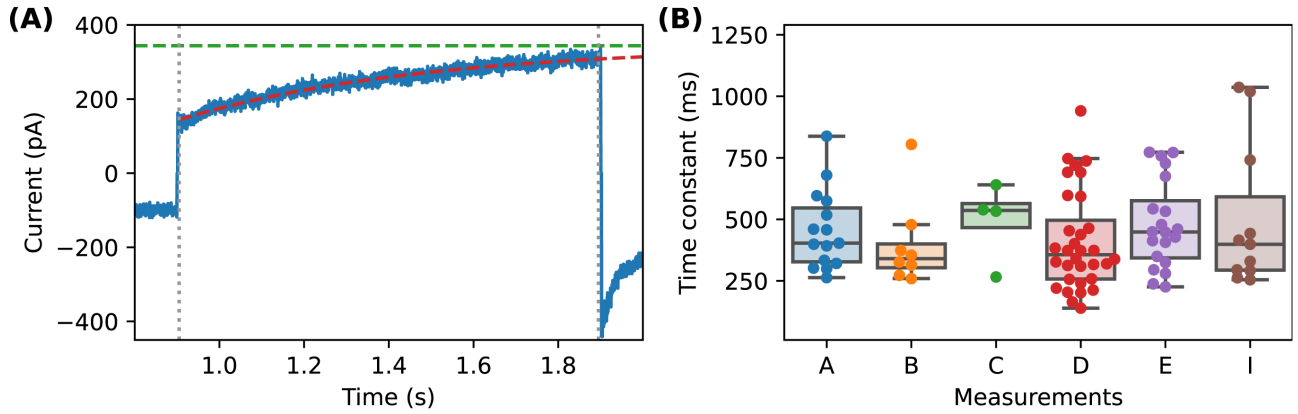

Figure 3. Time constants of the nonlinear time-dependent current in the presence of CaF_2. (**A**) Shows an example of the time constant estimation, where the blue line is the data, red is the fitted single exponential curve, the black dotted vertical lines indicate the region of the data used for fitting, and the green horizontal dashed line shows the estimated steady state current, as plotted in Figure 2 I-V plots. (**B**) Shows the histograms of the estimated time constants from various experiments, with Measurements A-E corresponding to the same set of experiments in Figure 2 and Measurement I the experiments in Figure 4.

as shown in Figures 2C and 2D, respectively. In the presence of CaF_2, all measurements exhibit the same nonlinear-in-voltage and time-dependent current. Figure 3 shows the time constants of the current during the first 40 mV step, all measurements shown in Figure 2 have similar distributions of time constants. Initially, these nonlinear currents were thought to be endogenous biological currents, given that they did not take the linear Ohmic form which is usual for leak currents:

$$I_{leak}=g_{leak}\times(V_m-E_{leak}), \quad (1)$$

where V_m is the membrane voltage, and g_{leak} and E_{leak} are the maximum conductance and reversal potential of the leak current. We illustrate the shape of a linear leak current given by Equation (1) by overlaying a fitted linear current (see Methods section) as an orange dashed line in Figure 2. Note that the leak current here is assumed to be through an imperfect seal, instead of current through some 'leak channels' in the cell. In contrast to the commonly-used human embryonic kidney (HEK) cells, CHO cells are thought to have relatively small endogenous currents (Yu & Kerchner, 1998).

The first question was whether the nonlinear time-dependent leftover current was endogenous current through native ion channels expressed in CHO cells. Figure 2E shows an empty well-plate experiment performed on the automated patch-clamp system (recorded as part of the study by Lei *et al.*, 2019a). That is, the experiments in Figure 2A–B & D were repeated without any biological cells. Since there were no membrane capacitances, no capacitance or series resistance compensations were applied. The recording shows a similar current, except with a much larger amplitude: the linear component appears to dominate – due to an open chip there is very low seal resistance and an enormous (nano- rather than pico-Amperes) linear leak current. But we see the same time constant for the nonlinear time-dependent part of the current (Figure 3B). This observation suggested the leftover current might not be endogenous CHO cell currents. The question then, is what could cause the nonlinear time-dependent current we observed?

Understanding the origin of the nonlinear time-dependent leftover current is crucial for accurate use of recordings. In the absence of any correction, the nonlinear current will obscure any real ion channel currents. If one uses a linear, non-time dependent leak correction (Equation (1)), the remaining nonlinear and time dependent current could be mistaken for a real ion channel current, contaminate the recording, and lead to an incorrect characterisation of ion channel gating.

One way to reduce this effect is to use post-blocker subtraction. That is, after measuring the complete current, apply a specific and approximately complete block of the current of interest (e.g. blocking hERG with dofetilide or E-4031) and remeasure the leftover current; the difference between the two recordings should be mainly the current of interest. We used this subtraction method in previous studies where we first observed this non-linear leftover current (Lei *et al.*, 2019a; Lei *et al.*, 2019b). This should remove the nonlinear time-dependent current from recordings, as well as any other currents that are not specifically blocked. But even then, with post-blocker subtraction the seal resistance could change over time (especially when a relatively long time period is needed for the blocker to have full effect, or when long protocols are required) and the subtraction method would not remove any changes in the nonlinear time-dependent leftover current. Studies without a specific-blocker subtraction method will certainly suffer from polluted currents.

In this study, we examine the origin of the observed nonlinear time-dependent leftover current.

Methods

The observation of the nonlinear time-dependent current in empty wells (Figure 2E) suggested this current might not be endogenous, and motivated further investigations using the F^- containing and Ca^{2+} containing solutions.

Voltage-clamp experiments were performed in the same way as the conventional manual patch experiments except the cell was replaced with a silicone elastomer ('SYLGARD'), as shown in Figure 1C.

A conventional manual patch-clamp system (HEKA EPC 10 USB Single, HEKA Elektronik GmbH, Lambrecht/Pfalz, Germany) was used for the voltage-clamp experiments. Similar to the no-cell automated experiments, since there was no cell membrane, no capacitance or series resistance compensations were applied. As the pipette tip was gradually moved closer to the silicone elastomer, a seal resistance in the range of 100–1000 MΩ could be obtained, similar to for example Lei *et al.* (2019a); Lei *et al.* (2019b), such that a magnitude of leak current could be measured that was similar to the biological measurements. Leak current between the pipette and the silicone with various patch-clamp solutions was measured and compared to the currents shown in Figure 2A–D.

All codes and data are freely available (see data and software availability Lei & Mirams (2021)).

Note that although we use the seal resistance as a reference of the quality of the seal, we avoid directly quoting the values to avoid overinterpretation. Seal resistance is defined as the inverse of the gradient of the (linear) I-V relationship, thus is usually estimated using two voltage steps in the I-V relationship, ideally when all ion channels are closed. However here we are examining a nonlinear time-dependent leak current, making direct interpretation of the seal resistance values inaccurate.

Patch-clamp solutions

Three sets of patch-clamp solutions were prepared: (1) a Ca^{2+} containing external solution; (2) a F^- containing internal solution; (3) a no-F^- internal solution. The concentrations of the solutions are given in Table 1, all substances were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Solutions (1) and (2) are the same as those used in Lei *et al.* (2019a); Lei *et al.* (2019b), which are very similar to other automated patch-clamp studies such as Kozek *et al.* (2020); Ng *et al.* (2020) and those suggested by Nanion for SyncroPatch platforms.

Silicone elastomer

The silicone elastomer (SYLGARD 184, The Dow Chemical Company) was prepared using a standard 10:1 ratio of base and catalyst. A thin layer of mixed elastomer was dispensed in 35 mm tissue culture dishes (product number 430165, Corning), and was cured at 60 °C.

Experimental procedure

Table 2 summarises the three sets of measurements that were performed. The currents were measured under a voltage-clamp protocol used in Lei *et al.* (2019a), known as the "staircase protocol", shown in Figure 2; a time series file for the protocol is provided (see Data Availability Lei & Mirams (2021)). The holding potential was set to 0 mV. Measurement I is the 'standard' configuration using solutions (1) and (2) (the same as in Figure 2), which aimed to reproduce the nonlinear

Table 1. The patch-clamp solutions used in the experiments. All concentrations are given in mM and product numbers refer to Sigma-Aldrich catalogue. Ca^{2+} containing (external) solution was titrated to pH 7.4 with HCl; F^- containing internal and no-F^- internal solutions were titrated to pH 7.2 with KOH.

Solutions Product number	NaCl S9625	KCl P4504	KF 449148	$MgCl_2$ 63069	$CaCl_2$ 21113	HEPES 54457	Glucose G8270	NMDG 66930	Sorbitol S1876	MgATP A9187	EGTA E4387
(1) Ca^{2+} containing	97.5	4	—	1	**2.05**	10	5	35	20	—	—
(2) F^- containing	10	10	**100**	—	—	10	—	—	—	—	20
(3) No F^-	—	130	—	1	—	10	—	—	—	5	5

Table 2. Summary of the three sets of voltage-clamp measurements performed using a manual patch-clamp system with silicone elastomers. Measurement III was performed by washing out the externally applied F^- containing solution in Measurement II with the no-F^- solution, the aim was that the measurements were done in the presence of the CaF_2 crystal but without F^- in solution.

Measurement	I	II	III
Internally applied solution	(2) F^- containing	(1) Ca^{2+} containing	(1) Ca^{2+} containing
Externally applied solution	(1) Ca^{2+} containing	(2) F^- containing	(3) No F^-

time-dependent leftover current we observed with real cells. Measurement II investigates the current's dependence on the ionic solutions by swapping the internal and external solutions. Measurement III forms a control experiment by washing out the F^- containing solution in Measurement II with the no-F^- solution; it was performed after Measurement II, such that the F^- containing solution was the external solution and could be easily changed.

Data analysis

We estimated g_{leak} and E_{leak} in Equation (1) using two voltage steps (−80 mV and −40 mV unless otherwise specified); g_{leak} was estimated by the ratio of the voltage difference and the mean current difference using the last 500 ms of the voltage steps, after which E_{leak} can be directly calculated from Equation (1) using one of the voltage steps (with mean and standard error across all recordings being E_{leak} = 8.14 ± 3.38 mV, but we do observe a fair amount of variation in E_{leak}). The steady state of the nonlinear time-dependent leak current at each voltage step was estimated by fitting a single exponential of the form: $a \times \exp(-(t - t_0) / \tau) + c$; where a, τ, c are the parameters to be fitted and t_0 is the starting time of the voltage step. Figure 3A shows an example of such an analysis. The first 5 ms at the beginning of each voltage step was ignored to avoid capacitive spikes. The parameter c is then the estimated steady state of the leak current of the given voltage step (as shown in Figure 3A and summarised for many cells in the right column of Figure 2), and the parameter τ is the time constant of the current (as summarised in Figure 3B). For the current-voltage relationships, each current was normalized relative to the linear leak estimate of the current within –120 mV and 40 mV. The analysis was performed in Python using NumPy/SciPy (Jones *et al.*, 2001); all the code for the analysis is provided (see software availability Lei & Mirams (2021)).

Results

The empty well-plate measurements in Figure 2E suggested that the nonlinear time-dependent current was non-biological current, and motivated further investigations using the F^- containing and Ca^{2+} containing solutions. Voltage-clamp experiments were repeated in manual patch with silicone elastomer (Figure 1C). Using this approach we ensured that: (1) the behaviour of the measured current was not caused by the planar-micro pore configuration (Figure 1B) or anything specific to the automated platform; and (2) recordings cannot be endogenous biological currents.

Measurement I

The recorded leak current for Measurement I is shown in Figure 4I, which was measured with the same solutions as in Figure 2 but with silicone elastomer. The leak current measured with silicone elastomer replicates the nonlinear time-dependent current we observed in CHO cells (Figure 2A–D). Not only were the size of the currents comparable (a few hundred pA), but also the steady state I-V curves were extremely similar to those seen at multiple sites, platforms and cells (Figure 2A–D). The measured leak current was time-dependent when it was held at a constant voltage, this is most noticeable for the outward (positive) current during an increase of voltage from 0 to +40 mV; the

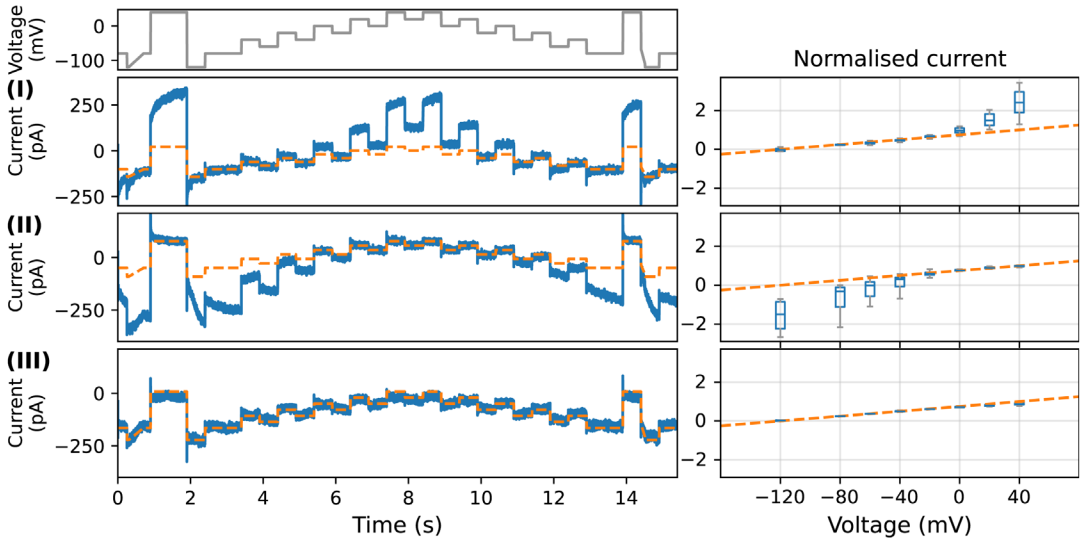

Figure 4. Manual patch-clamp recordings with CaF_2 solutions on silicone elastomer. Left: typical time series recordings under the staircase protocol (top panel) from Lei *et al.* (2019a); Right: the normalized current-voltage relationships (fitted with a single exponential function, approximating the steady state relation) in the boxplot for n repeats. Experimental recordings are shown in blue, and linear leak estimations are shown in dashed orange. (I)–(III) show the results of Measurements I–III in Table 2. (I) with internal and external solutions containing F^- and Ca^{2+} respectively (similar to those in Figure 2), with n = 13; (II) with internal and external solutions swapped, the nonlinear portions are now at negative rather than positive voltages (n = 4); (III) same conditions as (II) after F^- was washed out of the bath (n = 4) — the current becomes much closer to the expected linear leak given by Equation (1).

time constants of the current during the first +40 mV step were indistinguishable from those observed in the CHO cell experiments (Figure 3B). Furthermore, the I-V relationship (at plateau) was non-Ohmic (nonlinear).

Measurement II

In this set of measurements, we repeated the experiments but swapped the internal and external solutions in Measurement I, to investigate the current's dependence on the ionic solutions. Figure 4II shows the recorded leak current for Measurement II. Both the time-dependent part of the leak current and its nonlinear I-V relationship were reversed. Instead of a prominent outward (positive) time-dependent current during an increase of voltage from approximately 0 to +40 mV, a noticeable inward (negative) time-dependent current was produced during a decrease of voltage to approximately −80 to −120 mV. Moreover, the nonlinear I-V relationship (at plateau) changed from superlinear in Measurement I to sublinear, as shown in the right panels of Figure 4I–II. Note that, as the nonlinearity was thought to be caused by the inward time-dependent current at low voltage, the linear leak estimation (orange dashed line) in Figure 4II was fitted to two voltage steps at higher voltages (+40 mV and +60 mV).

Measurement III

Finally, as a control experiment, immediately following Measurement II during the same experiment the (now external) F^- containing solution was washed out and replaced with the no-F^- solution. Note that the measurement was performed by washing the external solution in Measurement II after the CaF_2 crystal was formed, and therefore the measurement should be in the presence of the CaF_2 crystal, although in some cases (but not all) the magnitude of the current became larger due to the wash perhaps indicating some loss of crystal and seal resistance. The results are shown in Figure 4III. The nonlinear I-V relationship and time-dependent dynamics of the leak current were almost entirely eliminated; linear leak current that follows Equation (1) was observed by simply removing F^- from the solution.

Discussion

We had observed a nonlinear and time-dependent current whilst taking recordings from CHO hERG1a cells in the presence of a hERG blocker, on an automated patch-clamp platform in the presence of calcium fluoride containing solutions. In this study, we investigated the origin of this 'leftover current' as it is crucial for accurately determining the kinetics of ion channel currents (Lei *et al.*, 2020). Experiments using a conventional manual patch-clamp setup on a silicone elastomer instead of a biological cell were performed with CaF_2-containing patch-clamp solutions.

Our results (Measurement I) show that it was possible to replicate the nonlinear time-dependent leftover current (Figure 2A–B) with manual 'no cell' (silicone elastomer) experiments (Figure 4I). Therefore the current was neither a feature of the automated patch clamp system nor an endogenous current from the (CHO) cells, and is predominantly a calcium-fluoride-dependent leak current through the imperfect seal. We then show that by interchanging the internal and external solutions (Measurement II), the time-dependence was retained but the nonlinear I-V relationship of the current was reversed (Figure 4I–II). This is evidence that the nonlinear time-dependent part of the leak current is determined by the two ionic solutions used. Finally, in Measurement III (Figure 4III), the nonlinear I-V relationship and time-dependent dynamics of the leak current were eliminated by washing out the externally applied F^- containing solution from Measurement II and replacing with the no-F^- solution. Observing a linear I-V relationship in Measurement III is indeed consistent with a previous study using Sylguard by Sachs & Qin (1993), whilst they also observed a nonlinear current in the presence of arginine. Note that the measurement was performed by washing the F^- containing solution in Measurement II after the crystal was formed, hence the nonlinear time-dependent leak current occurred as a consequence of the presence of the crystal *and* fluoride. This demonstrates that a requisite for the I-V nonlinearity and time-dependent behaviour of the leak current is the F^- containing internal solution used as part of a seal enhancer in automated patch-clamp systems.

We propose the following tentative hypothesis to explain the observed I-V nonlinearity and time-dependent behaviour of the leak current. Our leading conjecture is that the nonlinear time-dependent leak current is ordinary linear leak through imperfect seal with a conductance that changes nonlinearly and time-dependently with voltage. We speculate that the reason for this 'extra' current could be defects in the CaF_2 crystals: F^- has a higher mobility than Ca^{2+}, so F^- may preferentially move back into the cell from imperfections in the crystal when voltage is high. The extra defects in the crystal that result (sites missing fluoride, Huisinga, 1999) could allow a larger non-selective leak, forming the measured current. This process would reverse when voltage is low, with fluoride returning to 'plug the holes' in the crystal and reducing leak conductance, giving the observed asymmetry in leak current. This is a similar concept to e.g. positively charged polyamines causing block of inward rectifier potassium channels at positive potentials (Fakler *et al.*, 1995). That is, neither fluoride nor the polyamines contribute strongly to a measured current but cause its block. This hypothesis is consistent with the direction of the 'extra' current in the leak in both Measurements I and II.

Our findings have implications in methods for measuring and post-processing the recordings. The leak current has a nonlinear I-V relationship and time-dependence when it is held at a constant voltage, it is therefore important to subtract it off from the recordings such that a pure current of interest can be obtained. Due to its nonlinearity and time-dependence, the kinetics of the resulting current of interest (hERG1a current in our examples) could be undesirably affected if leak is not carefully removed: the fluoride-dependent leak current can shift the I-V curve of measured currents, alter observed time

constants, etc. Some manual patch clamp findings in the literature may also need to be re-examined in light of these observations.

In addition to the extra nonlinear time-dependent leak current, the use of intracellular fluoride and extracellular calcium as a seal enhancer could also give rise to other problems. Using intracellular fluoride, as a calcium chelator, prevents the use of calcium containing intracellular solutions, and fluoride is also a phosphatase inhibitor (Guranowski, 1990). Using extracellular calcium at higher concentrations than the physiological range can also alter channel gating (Ho *et al.*, 1998), at least partly due to membrane charge screening (McLaughlin *et al.*, 1971).

Since the form of this leak current is, to our knowledge, not very well studied; it is not possible to use the standard methods of estimating linear leak current to perform the correction. The standard methods involve a small leak step (change in voltage) at which the ion channel of interest is (nearly) closed. However, given the nonlinearity in the I-V relationship of this current, a voltage-current estimation across one range or pair of voltages would result in an incorrect estimation of the whole I-V relationship (see for example how the orange dashed lines missed the blue crosses in Figure 2 and Figure 4 right panels), and the time-dependent dynamics would not be captured. The best approach available at present is the widely-used block-and-subtract method, as used in our earlier studies (Lei *et al.*, 2019a; Lei *et al.*, 2019b). However, the seal can change over time (especially if a relatively long period is allowed for a blocker to take effect). In which case, the subtraction will not remove changes in the nonlinear time-dependent portion of the leak current, resulting in over- or under-subtracted nonlinear leak current. Therefore this study raises concerns about the effects and consequences of this nonlinear time-dependent leak/leftover current.

There are two obvious options to account for this current. Firstly, and ideally, we would completely remove this nonlinear time-dependent leak current by altering the ionic solutions. We have seen that the properties of the current depend on the concentration of F^- in the solutions (and will probably also depend on $[Ca^{2+}]$). Optimal concentrations for these ions may exist that are high enough to sufficiently enhance the seal, but low enough that the nonlinearity and time-dependence are not evident in recordings. Other salts such as BaF_2, $CaSO_4$, etc. should be tested to see whether they can enhance seals but remove this nonlinear time-dependent leak current, as well as examining their physiological effects on the cells (Tasaki & Takenaka, 1964). It may also be possible to restore a linear leak current by washing away the F^- ions after establishing a seal, as we did in Measurement III.

Secondly, further studies of this F^--dependent current could allow it to be modelled so well that it could be subtracted from recordings in post-processing in much the same way as the linear leak. But at a minimum this option would involve: a better characterisation of the time and voltage dependence of this leak current; its dependence on the concentration of F^- and/or Ca^{2+} in the solutions; dependence on the seal resistance; and testing that the current is predictable (and not, for example, a function of unmeasured quantities such as crystal size/thickness/volume). We anticipate that removing the current through alterations to the ionic solutions will be simpler and more reliable.

Conclusions

We recorded a nonlinear and time-dependent current in the presence of Ca^{2+} and F^- in patch clamp solutions (intended to form a CaF_2 seal enhancer). The same nonlinear time-dependent current was observed using a conventional manual patch-clamp setup in close proximity to a silicone elastomer to form a seal between 100 to 1000 MΩ. Therefore the current was determined to be mainly leak current through imperfect seal, and not endogenous biological currents. The nonlinear and time-dependent form of the leak current was caused by the presence of CaF_2 and could be eliminated by F^- washout after CaF_2 crystal formation.

Data availability

Underlying data

All datasets used in the publication are available at: https://github.com/CardiacModelling/nonlinear-time-dependent-leak.

A permanently archived version is available on Zenodo: https://doi.org/10.5281/zenodo.5571252 Lei & Mirams (2021)

This project contains the following underlying data:

- `data/protocol-staircaseramp.csv` — a time series trace of the voltage protocol.
- `data/silicone` and `data-rev/silicone` — a set of voltage-clamp timeseries data in HEKA format for Measurements I, II and III; as plotted in Figure 4)
- `data/cho-cell`, `data-rev/cho-herg` and `data-rev/cho-herg-2` — a set of CHO-hERG cell voltage-clamp time-series data taken from (Lei *et al.*, 2019a), as plotted in Figure 2.
- `data-rev/cho-empty`and`data-rev/cho-empty-auto` — a set of untransfected CHO cell voltage-clamp time-series data as plotted in Figure 2.
- `data/no-cell` and `data-rev/no-cell` — a set of empty well-plate voltage-clamp time-series data taken from (Lei *et al.*, 2019a), as plotted in Figure 2.

A description of other files, including python scripts to read and plot these data, is available in the repository Readme file.

Software availability

Source code is also available from: https://github.com/CardiacModelling/nonlinear-time-dependent-leak and was archived at time of publication: https://doi.org/10.5281/zenodo.5571252 Lei & Mirams (2021)

License: BSD 3-Clause

References

Bell DC, Fermini B: **Use of automated patch clamp in cardiac safety assessment: Past, present and future perspectives.** *J Pharmacol Toxicol Methods.* 2021; **110**: 107072.
PubMed Abstract | Publisher Full Text

Billet A, Froux L, Hanrahan JW, *et al.*: **Development of automated patch clamp technique to investigate cftr chloride channel function.** *Front Pharmacol.* 2017; **8**: 195.
PubMed Abstract | Publisher Full Text | Free Full Text

Elkins RC, Davies MR, Brough SJ, *et al.*: **Variability in high-throughput ion-channel screening data and consequences for cardiac safety assessment.** *J Pharmacol Toxicol Methods.* 2013; **68**(1): 112–122.
PubMed Abstract | Publisher Full Text | Free Full Text

Fakler B, Brändle U, Glowatzki E, *et al.*: **Strong voltage-dependent inward rectification of inward rectifier K^+ channels is caused by intracellular spermine.** *Cell.* 1995; **80**(1): 149–54.
PubMed Abstract | Publisher Full Text

Gertler TS, Thompson CH, Vanoye CG, *et al.*: **Functional consequences of a *KCNT1* variant associated with status dystonicus and early-onset infantile encephalopathy.** *Ann Clin Transl Neurol.* 2019; **6**(9): 1606–1615.
PubMed Abstract | Publisher Full Text | Free Full Text

Guranowski A: **Fluoride is a strong and specific inhibitor of (*asymmetrical*) Ap_4A hydrolases.** *FEBS Lett.* 1990; **262**(2): 205–8.
PubMed Abstract | Publisher Full Text

Ho WK, Kim I, Lee CO, *et al.*: **Voltage-dependent blockade of HERG channels expressed in *Xenopus* oocytes by external Ca^{2+} and Mg^{2+}.** *J Physiol.* 1998; **507**(Pt 3): 631–8.
PubMed Abstract | Publisher Full Text | Free Full Text

Hodgkin AL, Huxley AF: **A quantitative description of membrane current and its application to conduction and excitation in nerve.** *J Physiol.* 1952; **117**(4): 500–544.
PubMed Abstract | Publisher Full Text | Free Full Text

Huisinga M: **Ultraviolet photoelectron spectroscopy and electron stimulated desorption from CaF_2.** PhD thesis, The Free University of Berlin, 1999.
Publisher Full Text

Jones E, Oliphant T, Peterson P, *et al.*: **SciPy: Open source scientific tools for Python**. 2001; [Online; accessed 2020-05-30].
Reference Source

Kang SK, Vanoye CG, Misra SN, *et al.*: **Spectrum of $k_v2.1$ dysfunction in *KCNB1*-associated neurodevelopmental disorders.** *Ann Neurol.* 2019; **86**(6): 899–912.
PubMed Abstract | Publisher Full Text | Free Full Text

Kostyuk PG, Krishtal OA, Pidoplichko VI: **Effect of internal fluoride and phosphate on membrane currents during intracellular dialysis of nerve cells.** *Nature.* 1975; **257**(5528): 691–693.
PubMed Abstract | Publisher Full Text

Kozek KA, Glazer AM, Ng CA, *et al.*: **High-throughput discovery of trafficking-deficient variants in the cardiac potassium channel $K_v11.1$.** *Heart Rhythm.* 2020; **17**(12): 2180–2189.
PubMed Abstract | Publisher Full Text | Free Full Text

Kramer J, Himmel HM, Lindqvist A, *et al.*: **Cross-site and cross-platform variability of automated patch clamp assessments of drug effects on human cardiac currents in recombinant cells.** *Sci Rep.* 2020; **10**(1): 5627.
PubMed Abstract | Publisher Full Text | Free Full Text

Lei CL, Wang K, Clerx M, *et al.*: **Tailoring mathematical models to stem-cell derived cardiomyocyte lines can improve predictions of drug-induced changes to their electrophysiology.** *Front Physiol.* 2017; **8**: 986.
PubMed Abstract | Publisher Full Text | Free Full Text

Lei CL, Clerx M, Gavaghan DJ, *et al.*: **Rapid characterization of hERG channel kinetics I: using an automated high-throughput system.** *Biophys J.* 2019a; **117**(12): 2438–2454.
PubMed Abstract | Publisher Full Text | Free Full Text

Lei CL, Clerx M, Beattie KA, *et al.*: **Rapid characterization of hERG channel kinetics II: temperature dependence.** *Biophys J.* 2019b; **117**(12): 2455–2470.
PubMed Abstract | Publisher Full Text | Free Full Text

Lei CL, Clerx M, Whittaker DG, *et al.*: **Accounting for variability in ion current recordings using a mathematical model of artefacts in voltage-clamp experiments.** *Philos Trans A Math Phys Eng Sci.* 2020; **378**(2173): 20190348.
PubMed Abstract | Publisher Full Text | Free Full Text

Lei CL, Mirams G: **A nonlinear and time-dependent leak current in the presence of calcium fluoride patch-clamp seal enhancer.** 2021.
http://www.doi.org/10.5281/zenodo.5571252

Li T, Lu G, Chiang EY, *et al.*: **High-throughput electrophysiological assays for voltage gated ion channels using syncropatch 768PE.** *PLoS One.* 2017; **12**(7): e0180154.
PubMed Abstract | Publisher Full Text | Free Full Text

Lojkner LD, Lindqvist A, Sauter DR, *et al.*: **Seal enhancer.** (United States Patent No. US 2019/0383792 A1). *United States Patent.* 2019.
Reference Source

Maltsev VA, Undrovinas AI: **Cytoskeleton modulates coupling between availability and activation of cardiac sodium channel.** *Am J Physiol.* 1997; **273**(4): H1832–40.
PubMed Abstract | Publisher Full Text

McLaughlin SG, Szabo G, Eisenman G: **Divalent ions and the surface potential of charged phospholipid membranes.** *J Gen Physiol.* 1971; **58**(6): 667–87.
PubMed Abstract | Publisher Full Text | Free Full Text

Ng CA, Perry MD, Liang W, *et al.*: **High-throughput phenotyping of heteromeric human ether-à-go-go-related gene potassium channel variants can discriminate pathogenic from rare benign variants.** *Heart Rhythm.* 2020; **17**(3): 492–500.
PubMed Abstract | Publisher Full Text

Rugiero F, Mistry M, Sage D, *et al.*: **Selective expression of a persistent tetrodotoxin-resistant Na^+ current and NaV1.9 subunit in myenteric sensory neurons.** *J Neurosci.* 2003; **23**(7): 2715–25.
PubMed Abstract | Publisher Full Text | Free Full Text

Sachs F, Qin F: **Gated, ion-selective channels observed with patch pipettes in the absence of membranes: novel properties of a gigaseal.** *Biophys J.* 1993; **65**(3): 1101–7.
PubMed Abstract | Publisher Full Text | Free Full Text

Sakmann B, Neher E: **Patch clamp techniques for studying ionic channels in excitable membranes.** *Annu Rev Physiol.* 1984; **46**(1): 455–472.
PubMed Abstract | Publisher Full Text

Tasaki I, Takenaka T: **Effects of various potassium salts and proteases upon excitability of intracellularly perfused squid giant axons.** *Proc Natl Acad Sci U S A.* 1964; **52**(3): 804–810.
PubMed Abstract | Publisher Full Text | Free Full Text

Toh MF, Brooks JM, Strassmaier T, *et al.*: **Application of high-throughput automated patch-clamp electrophysiology to study voltage-gated ion channel function in primary cortical cultures.** *SLAS Discov.* 2020; **25**(5): 447–457.
PubMed Abstract | Publisher Full Text

Volkers L, Kahlig KM, Das JH, *et al.*: **Febrile temperatures unmask biophysical defects in Nav1.1 epilepsy mutations supportive of seizure initiation.** *J Gen Physiol.* 2013; **142**(6): 641–653.
PubMed Abstract | Publisher Full Text | Free Full Text

Vanoye CG, Desai RR, Fabre KL, *et al.*: **High-throughput functional evaluation of *kcnq1* decrypts variants of unknown significance.** *Circ Genom Precis Med.* 2018; **11**(11): e002345.
PubMed Abstract | Publisher Full Text | Free Full Text

Wang DW, George AL Jr, Bennett PB: **Comparison of heterologously expressed human cardiac and skeletal muscle sodium channels.** *Biophys J.* 1996; **70**(1): 238–45.
PubMed Abstract | Publisher Full Text | Free Full Text

Wendt DJ, Starmer CF, Grant AO: **Na channel kinetics remain stable during perforated-patch recordings.** *Am J Physiol.* 1992; **263**(6 Pt 1): C1234–40.
PubMed Abstract | Publisher Full Text

Yu SP, Kerchner GA: **Endogenous voltage-gated potassium channels in human embryonic kidney (HEK293) cells.** *J Neurosci Res.* 1998; **52**(5): 612–617.
PubMed Abstract | Publisher Full Text

Open Peer Review

Current Peer Review Status: ✔ ? ✔ ✔

Version 2

Reviewer Report 16 November 2021

https://doi.org/10.21956/wellcomeopenres.19142.r46896

✔ **Carlos Guillermo Vanoye**
Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

The authors addressed my concerns, I have only minor text editing comments:

In the Abstract line 2, it reads as if a leak occurs when the seal is not perfectly insulated from the bath. Please rewrite to mean that a leak occurs when the seal does not perfectly insulate the bath (extracellular) from the internal solution.

.Abstract line 14: “non-linear-in-voltage” is confusing, please re-write.

Page 3, column 2, paragraph 4: Line 5: “Figure 2’s” should read “Figure 2”. Line 8: use “non-transfected CHO cells” instead of non-hERG-transfected WT CHO cells.

Page 5, paragraph 1: Line 6: “endogenous biological currents” should read “endogenous currents”.

Page 6 column 1, Methods paragraph 1, line 2 “empty wells..” should read “cell-free wells”.

Page 7, column 1 paragraph 1, line 1: “with real cells should read “with cells”

Page 7 column 2, Results paragraph 1: “..empty well-plate..” should read “cell-free well-plate”.

Page 8, column 2, paragraph 1: “..(CHO) cells.” should read “..CHO cells.”

The use of the term “no biological cells” throughout the text is misleading because Model Cells were not used when CHO cells were not recorded. I would suggest to use the terms “cell-free” or “in the absence of cells” instead.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ion channels, electrophysiology, molecular biology, cell physiology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 15 November 2021

https://doi.org/10.21956/wellcomeopenres.19142.r46897

Gildas Loussouarn
CNRS, INSERM,the thorax institute, University of Nantes, Nantes, France

I approve the manuscript but I have still a few minor comments regarding Version 2, essentially text edits.

Regarding my previous comment: I agree that in the conditions now described, the time-dependent current can not be due to Rs compensation in Figure 2E (no cells).
But do the authors have another explanation for the fact that the current does not show any clear time dependence when voltage changes from 0 to +40mV in the middle of panel (E), but only when voltage changes from -80 mV to +40mV (left and right of panel E)? In other conditions, time dependence is visible in both voltage steps (-80 to +40 mV and 0 to +40mV).

Abstract : A leak current to ground occurs whenever the seal between a pipette and cell (or internal solution and cell in high-throughput machines) is not perfectly insulated from the bath (extracellular) solution.
Something is wrong with this sentence: I would say "A leak current to ground occurs whenever the seal does not insulate the intra-cellular solution (in the pipette in manual patch or the chip in high-throughput machines) from the extracellular (bath) solution."

Page 3: Studies have compared manual patch clamping with automated patch clamping data, and showed that their performances are similar (Billet *et al.*, 2017).
I think this sentence is misleading, 'similar performance' may me interpreted as the quantity of data rather than quality of data.

Page 3 : as shown in Figure 2's (right panels) blue boxplot
there are several 's that are not necessary, or even relevant.

Page 3: Measurements were repeated with non-hERG transfected wild-type CHO cells using a manual patch-clamp system.
I would replace with "non-transfected CHO cells"

Page 7: Eleak can be directly calculated from Equation (1) using one of the voltage steps (with mean and standard error across all recordings being Eleak = 8.14 ± 3.38 mV.

Is this value close to liquid junction potential?

Page 7: this is most noticeable for the outward (positive) current during an increase of voltage from 0 to +40 mV.
Should it be "from -80mV to +40mV"? The time dependence is the most noticeable at this voltage step.

Figure 4. Right: the normalized current-voltage relationships (fitted with a single exponential function, approximating the steady state relation)
Not clear to me: it is not the I/V relationship that has been fitted. Please rephrase.

Page 8. Moreover, the nonlinear I-V relationship (at plateau) changed from superlinear in Measurement I to sublinear, as shown in the right panels of Figure 4I–II.
Is it really "moreover" since it is a direct consequence of the above replacement of the outward current by the inward current?

Page 8 Our results (Measurement I) show that it was possible to replicate the nonlinear time-dependent leftover current (Figure 2A–B) with manual 'no cell' (silicone elastomer) experiments (Figure 4I).
manual 'no cell' is not clear, please rephrase.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electrophysiology, patch-clamp.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 November 2021

https://doi.org/10.21956/wellcomeopenres.19142.r46895

Marc Rogers
Metrion Biosciences Limited, Cambridge, UK

The additional data (experiments with untransfected cells, N replicates) is helpful, as-is the rewording of several text sections.

However, I would still like to highlight several places where the focus on automated patch clamp (APC) remains and is misleading or potentially biased:

Abstract
The authors neglect to include new data showing that non-linear currents are observed in

untransfected CHO cells under manual patch (e.g after sentence #6 'However,...automated patch clamp platform', or sentence #7 'We performed... leak current'.

Discussion
p2, last sentence could also include the fact that they see a similar non-linear current with cell-based manual patch recordings as well.

Although minor, I would prefer that the authors consider a final edit to warrant full acceptance of this review and proceed to indexing of a final version.

Competing Interests: I am a shareholder and non-executive director of Metrion Biosciences Ltd, a UK-based commercial entity supplying patch clamp screening services using manual and automated patch platforms. We are an independent company and use multiple patch clamp platforms from major vendors without preference or bias.

Reviewer Expertise: Manual patch clamp, automated patch clamp, drug discovery screening, pharmacology, ion channel biophysics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 30 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39340

?

Carlos Guillermo Vanoye
Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

In the present manuscript, Lei *et al.* describe a rectifying, time-dependent leak current that is fluoride-dependent. This is well written manuscript with very interesting results. Their observation is important because some high throughput systems require the presence of fluoride to enhance seal formation. And the presence of this current, if not corrected properly, could contaminate ion channel recordings complicating results interpretation.
Comments:

1. Were the current traces shown in Figs 2 and 3 recorded with capacitance and series resistance compensation turned on? The compensations may affect the shape of the observed current.

2. The seal resistance values for the recordings shown in Figs. 2 and 3 are not given. Those values would allow the reader to interpret the amplitude of the leak current shown. This information is critical specially when comparing the results in Figure 3.II and Figure 3.III.

3. Each of the I-Vs shown in Figures 2 and 3 appear to be derived from only one recording. Please provide data from multiple wells (Fig 2) and patches (Fig 3) and show means, standard errors (or standard deviations) and statistical significance. The conclusions would be supported by providing I-Vs derived from multiple observations not just from one.

4. The slope of the outward current in Fig.2A and 2B is steeper than the one shown in Fig 2C. It is assumed that no currents are going through the cell membrane on those recordings but this may not be the case. Is the block of hERG1a by E-4031 100%? There could also be chloride channels (Gill *et al*, 2006)[1] that may be carry the observed current. The conclusion that there is no current going through ion channels in those recordings would be strengthened if cesium was used instead of potassium (or using non-transfected CHO-K1 cells) and chloride channel blockers were added.

5. Regarding the results shown in Figure 3, the authors should compare their observations to those previously reported by Sachs and Qin (1993)[2]. The previous results showed ion-selective currents in the absence of CaF_2 using a similar approach (glass pipette plus Sylgard).

6. Is the linearity of the IV curve shown in Fig. 3.III due to the absence of fluoride or to the large drop in seal resistance? Please provide pre- and post-wash seal resistance values. Also multiple recordings ("patches") would be recommended.

7. Is the drop in resistance due to the loss of fluoride (and presumably CaF_2 crystals)? Or to the pipette and Sylgard moving apart during the wash? Performing a wash with a fluoride-containing solution could answer this question.

References

1. Gill S, Gill R, Xie Y, Wicks D, et al.: Development and validation of HTS flux assay for endogenously expressed chloride channels in a CHO-K1 cell line.*Assay Drug Dev Technol*. 2006; **4** (1): 65-71 PubMed Abstract | Publisher Full Text
2. Sachs F, Qin F: Gated, ion-selective channels observed with patch pipettes in the absence of membranes: novel properties of a gigaseal. *Biophysical Journal*. 1993; **65** (3): 1101-1107 Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ion channels, electrophysiology, molecular biology, cell physiology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 26 Oct 2021

Gary Mirams, University of Nottingham, Nottingham, UK

We thank the reviewer for the insightful and thorough review. This post relates to the changes introduced in Version 2 of the manuscript. The reviewer's comments are in bold and italic, with our point-by-point response below.

Comments:
Were the current traces shown in Figs 2 and 3 recorded with capacitance and series resistance compensation turned on? The compensations may affect the shape of the observed current.

Indeed the compensation may affect the shapes of the observed current, but given the size of the current, membrane capacitance, and series resistance, both the time dependence (time constant) and the size of the effect (current size) due to membrane capacitance and series resistance are in a completely different scale (and in fact opposite direction) compared to the nonlinear time-dependent current that was observed. A comparison of the capacitance and series resistance effect is shown in Lei *et al.*, 2020 [1].
Furthermore, the compensations were turned on for the measurements in Figure 2. This should minimise the changes to the shape of the observed current due to cell membrane capacitance and series resistance. Whilst the compensations were turned off for the measurements in Figure 3 (new Figure 4) as silicone elastomers do not have a membrane capacitance which would not give rise to any time-dependent effect.
We have added the following text to Introduction:
"*[Figure 2A shows an automated patch (Nanion SyncroPatch 384PE) recording of the leftover current measured on Chinese hamster ovary (CHO) cells transfected with the human Ether-à-go-go-Related Gene (hERG)1a after applying a hERG-specific blocker (in this case 0.5 μM of E-4031) at 25°C,] with capacitance and 80% series resistance compensations according to manufacturer's settings.*"

"*[That is, the experiments in Figure 2A–B & D were repeated without adding in any biological cells.] Since there were no membrane capacitance, no capacitance and series resistance compensations were applied.*"
And to Methods:
"*Similar to the no-cell experiments, since there were no cell membrane capacitance, no capacitance and series resistance compensations were applied.*"
[1] Lei et al. 2020. Accounting for variability in ion current recordings using a mathematical model of artefacts in voltage-clamp experiments. *Phil. Trans. R. Soc. A*. 378:20190348. https://doi.org/10.1098/rsta.2019.0348

The seal resistance values for the recordings shown in Figs. 2 and 3 are not given. Those values would allow the reader to interpret the amplitude of the leak current shown. This information is critical specially when comparing the results in Figure 3.II and Figure 3.III.

This is an interesting question as to whether we are able to accurately measure (or rather estimate) the seal resistance values. Whether we can safely use the definition of seal resistance is perhaps a better question. Seal resistance is defined as the inverse of the gradient of the I-V relationship, thus is measured (or estimated) for example using two points (i.e. two voltage steps) in the I-V relationship ideally when all ion channels are closed. In Figures 2 and 3, we can see that the I-V relationship of the leak current is non-linear, and that we cannot easily separate between the linear leak due to the imperfect seal (hence defining the seal resistance values) and the nonlinear time-dependent part of the leak current due to the crystals, hence it would be difficult and inaccurate to simply quote the seal resistance values for interpretation.
We have added this explanation to the main text in Methods, reads as:
"*Note that although we use the seal resistance as a reference of the quality of the seal, we avoid directly quoting the values for overinterpretation. Seal resistance is defined as the inverse of the gradient of the (linear) I-V relationship, thus is usually estimated using two voltage steps in the I-V relationship, ideally when all ion channels are closed. However here we are examining a nonlinear time-dependent leak current, making direct interpretation of the seal resistance values inaccurate.*"

Each of the I-Vs shown in Figures 2 and 3 appear to be derived from only one recording. Please provide data from multiple wells (Fig 2) and patches (Fig 3) and show means, standard errors (or standard deviations) and statistical significance. The conclusions would be supported by providing I-Vs derived from multiple observations not just from one.

Thanks for the suggestion. Multiple repeats of the experiments were included, and boxplots of the I-V curves are shown in all figures. The results were the same as the previously shown example, therefore we did not change the conclusion that we drew from the observations.

The slope of the outward current in Fig.2A and 2B is steeper than the one shown in Fig 2C. It is assumed that no currents are going through the cell membrane on those recordings but this may not be the case. Is the block of hERG1a by E-4031 100%? There could also be chloride channels (Gill et al, 2006)1 that may be carrying the observed current. The conclusion that there is no current going through ion channels in those recordings would be strengthened if cesium was used instead of potassium (or using non-transfected CHO-K1

cells) and chloride channel blockers were added.

Thanks for the suggestion. Additional experiments was carried out with non-transfected CHO cells using a manual patch clamp system and the automated patch clamp platform with the same Ca-F containing solution, which is shown in new Figure 2C and D, showing the same nonlinear current as observed in Figure 2A and B. The difference between the slopes (Figure 2A-D and Figure 2E) was due to the ratio between the linear leak and the 'extra' time-dependent leak due to CaF2, as the no-cell experiment is expected to have a much larger linear leak. To confirm that they are the same type of time-dependent leak, a new Figure 3 is included, showing the time constants of the currents (for Figure 2A-D, Figure 2E and Measurement I) are the same.

Regarding the results shown in Figure 3, the authors should compare their observations to those previously reported by Sachs and Qin (1993)2. The previous results showed ion-selective currents in the absence of CaF2 using a similar approach (glass pipette plus sylgard).

Thank you for the reference, indeed it is an interesting comparison. They have shown a nonlinear type of leak current when using 400mM NaCl for the pipette solution and 400mM arginine for the bath solution. Although for all other non-organic solution combinations they observed a 'typical' linear leak current as we did in our Measurement III. We have now included a mention of this paper at the start of the discussion:
"*[...]; observing a linear I-V relationship in Measurement III is indeed consistent with a previous study using Sylguard by Sachs and Qin (1993), whilst they also observed a nonlinear current in the presence of arginine.*"

Is the linearity of the IV curve shown in Fig. 3.III due to the absence of fluoride or to the large drop in seal resistance? Please provide pre- and post-wash seal resistance values. Also multiple recordings ("patches") would be recommended. Is the drop in resistance due to the loss of fluoride (and presumably CaF2 crystals)? Or to the pipette and Sylgard moving apart during the wash? Performing a wash with a fluoride-containing solution could answer this question.

This was a weakness of the original manuscript, the wash-in of a fresh solution for Measurement II could have disturbed the CaF2 crystal and created more large holes for a linear leak current in parallel with a remaining nonlinear current. However, upon repeating the Sylguard Meaurement III to improve the '*n*' for this revision we did observe cells where there was very little change in apparent seal resistance and we still saw the nonlinear leak disappear, as reflected in the new figures, pointing to the disappearance of the nonlinear current in the absence of fluoride rather than the addition of a much larger linear leak.

Competing Interests: N/A

Reviewer Report 15 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39338

?

Marc Rogers
Metrion Biosciences Limited, Cambridge, UK

Lei *et al.* expand on a historical automated patch clamp (APC) study of hERG current kinetics with a replicate APC study from a collaborator laboratory, to confirm the evidence for a nonlinear time- and voltage-dependent current component of unknown origin that would be problematic to subtract or remove using traditional pharmacological or biophysical techniques. In an effort to determine the basis of this non-linear current they emulate it using manual patch pipette recordings from an artificial silicone 'cell', concluding that the combination of a high (internal) concentration of fluoride ions and a physiological concentration of (external) Ca2+ ions either side of a membrane seal produces a CaF crystal that can lead to non-linear current in APC recordings.

- Is the work clearly and accurately presented and does it cite the current literature?

Yes

- Is the study design appropriate and does the work have academic merit?

Partly – lack of cell-based data to back-up claims made using artificial silicone cell.

- Are sufficient details of methods and analysis provided to allow replication by others?

Yes

- If applicable, is the statistical analysis and its interpretation appropriate?

Partly – additional replicates, means and statistical variation data would be useful.

- Are all the source data underlying the results available to ensure full reproducibility?

Yes

- Are the conclusions drawn adequately supported by the results?

Partly - see below.

Starting in the Abstract, the authors fail to adequately discriminate and make clear that their findings, and technical data, do not apply to all automated patch clamp platforms. It is neither fair nor accurate to paint all APC platforms with the same brush, as seems common in some academic papers and comments on this technology. Each APC platform is different, and their experience and main point about the combination of internal fluoride and high external divalent 'seal enhancer' being obligate to achieving gigaohm seals primarily applies to a single APC platform manufacturer.

The second major issue is that the authors seem to suggest that a combination of high internal fluoride and external divalent cations is required to acquire gigaohn seals on APC platforms, and again this is inaccurate and may reflect their lack of experience with multiple platforms. It is true that some APC platforms may rely on high internal fluoride to achieve a high frequency of gigaohn seals, but this is neither obligate nor common these days, and it is actually contraindicated to combine high internal fluoride and elevated external divalents for many APC platforms.

Also, the authors just tested a single combination of internal F and external Ca^{2+} (Table 1), but should and could have looked at several different combinations and concentrations (as suggested

in the Discussion) to determine the true source and magnitude of the CaF effect. In addition, many groups can achieve gigaseals without using >100 mM internal fluoride through a combination of optimised cell culture and experimental conditions, and biocompatible chip substrates. Thus, the statements in the Abstract that "*With automated platforms it can be difficult to obtain such a high seal resistance between the intra- and extra-cellular solutions*" and "*One suggested method to alleviate this problem is using an F containing internal solution together with a Ca containing external solution — so that a CaF crystal forms when the two solutions meet which 'plugs the holes' to enhance the seal resistance*", and the schematic and legend to Fig. 1B, are inaccurate and incorrect.

As an APC user myself and a decades long patch clamper, I also have difficulty accepting the implication that the use of high internal fluoride and even low mM external divalents is a 'trick' solely employed for gigaseal APC recordings. I have lost track of the number of peer reviewed publications on voltage-gated Nav channels from leading academic groups, for example, that use this exact same recipe for their manual patch recordings, largely to ensure high resistance high fidelity recordings. Fig. 1A ignores this well-known tradition, assigning manual patch gigaseal resistances to the pipette glass-membrane tight seal alone. Thus, I would expect that a similar non-linear leak phenomenon would also be observable in many typical manual patch clamp recordings, but the authors notably did not run this experiment, and instead opted for a cell-free silicone-based biophysical manual patch pipette test. Thus, a claim or suggestion for 'correction' or close inspection of APC recordings employing a CaF effect would thus equally apply to a great number of past and future manual patch clamp datasets, but the authors do not include this possibility in their Abstract or Conclusions.

The authors assume that all time- and voltage-dependent, exogenously expressed hERG channel current is removed in the presence of the pharmacological blocker, but do not provide evidence that this is the case under their recording conditions. By inference they suggest this is the case, and thus the similarity between the remaining non-linear outward leak current (Fig. 2A, B) and the open chip APC recording (Fig. 2C) is due to the CaF2 effect, rather than remaining hERG conductance.

Similarly, the authors cite a reference on p4 that CHO cells have 'little endogenous current', but could have easily determined this empirically using wildtype or non-transfected cells and the same recording conditions and APC platforms used in the present study. Both sets of additional cell-based experiments would have bolstered their silicone 'cell' dataset, removing two possible related explanations for the APC cell non-linearity (leftover hERG and/or endogenous conductances, both of which would be expected to be time-dependent and non-linear at positive voltages), and strengthening their main claim about a non-linear CaF leak effect without relying on a simple 'they look the same' argument.

Obviously there is a CaF-mediated non-linear biophysical phenomenom seen in the silicone-cell manual patch experiments, but acceptance of this manuscript and their (modified/clarified) claims about this affecting certain APC platform recordings requires actual cell-based data to compliment their historical APC datasets.

Also, their Discussion suggestions at the bottom on p7 are somewhat out-of-date, as many of these options have already been explored by experienced APC users (e.g. reducing Fl and Ca concentrations, use of alternate cations).

Finally, the language in the Conclusions needs to be re-worded to limit their claims to a certain type of APC platform (i.e. remove the plural to 'APC platforms'). Also, the Conclusion stating that the CaF non-linear leak current is not due to endogenous (or non-blocked hERG) conductances is also not bourne out by the lack of manual patch clamp cell-based experimental data in this study, as outlined above.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Manual patch clamp, automated patch clamp, drug discovery screening, pharmacology, biophysics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 26 Oct 2021

Gary Mirams, University of Nottingham, Nottingham, UK

We thank the reviewer for their insightful review. This post relates to the changes introduced in Version 2 of the manuscript. The reviewer's comments are in bold and italic with our point-by-point responses below.

To address the reviewer's comments topic-by-topic, we have slightly re-ordered the reviewer's comments here, but include them all.

Starting in the Abstract, the authors fail to adequately discriminate and make clear that their findings, and technical data, do not apply to all automated patch clamp platforms. It is neither fair nor accurate to paint all APC platforms with the same brush, as seems

common in some academic papers and comments on this technology. Each APC platform is different, and their experience and main point about the combination of internal fluoride and high external divalent 'seal enhancer' being obligate to achieving gigaohm seals primarily applies to a single APC platform manufacturer.

We completely agree that this is not a problem with APC platforms per-se. We were attempting a narrative style to start with where we first observed the problem, but agree that it perhaps confused the message. We have substantially re-worked the text to highlight that this is a problem with the solutions regardless of platform, rather than the platform.

The second major issue is that the authors seem to suggest that a combination of high internal fluoride and external divalent cations is required to acquire gigaohn seals on APC platforms, and again this is inaccurate and may reflect their lack of experience with multiple platforms. It is true that some APC platforms may rely on high internal fluoride to achieve a high frequency of gigaohm seals, but this is neither obligate nor common these days, and it is actually contraindicated to combine high internal fluoride and elevated external divalents for many APC platforms. In addition, many groups can achieve gigaseals without using >100 mM internal fluoride through a combination of optimised cell culture and experimental conditions, and biocompatible chip substrates. Thus, the statements in the Abstract that "With automated platforms it can be difficult to obtain such a high seal resistance between the intra- and extra-cellular solutions" and "One suggested method to alleviate this problem is using an F containing internal solution together with a Ca containing external solution — so that a CaF crystal forms when the two solutions meet which 'plugs the holes' to enhance the seal resistance", and the schematic and legend to Fig. 1B, are inaccurate and incorrect.

We have substantially reworded the introductory text to remove the focus on APC platforms. Although not all APC experiments include the fluoride seal enhancer it has been very widely used; a recent cross-site and cross-platform comparison of ion channel screening for multiple voltage-gated cardiac ion channels was undertaken (Kramer et al., 2020). Whilst the data were anonymised, that publication details screening at 17 sites for hERG, Nav1.5 and CaV1.2 on 5 APC platforms, for a total of 39 assays being run (not all sites screened all channels). Non-proprietary internal solution data were available for 16/17 sites, 37/39 assays, 4/5 platforms. In our experiments we used 2.05mM [Ca^{2+}], 1mM [Mg^{2+}] and 100mM [F^{-}]. In the supplement of Kramer et al. (2020) we can see that 30/37 (81%) of cardiac ion channel screening assays used substantial amounts of fluoride (> 30mM) in the internal solution together with calcium in the external solution (at >1mM); and many used [F^{-}]>=120mM, whilst using similar amounts of Ca2+ to us (and most used Mg^{2+} as well). Only 2 of 18 sites used no fluoride in any of their channel assays (but neither of these screened all three channels), and it was used on all 4 APC platforms in at least some of the channel assays.

Also, the authors just tested a single combination of internal F and external Ca2+ (Table 1), but should and could have looked at several different combinations and concentrations (as suggested in the Discussion) to determine the true source and magnitude of the CaF effect.

In this short Research Note we are just hoping to draw attention to the problem, which we

have not seen reported before, rather than propose a solution, so we have not examined the many factors which could influence the current size, but we have performed many more repeats of the original conditions to bolster the existing findings.

The authors assume that all time- and voltage-dependent, exogenously expressed hERG channel current is removed in the presence of the pharmacological blocker, but do not provide evidence that this is the case under their recording conditions. By inference they suggest this is the case, and thus the similarity between the remaining non-linear outward leak current (Fig. 2A, B) and the open chip APC recording (Fig. 2C) is due to the CaF2 effect, rather than remaining hERG conductance. Similarly, the authors cite a reference on p4 that CHO cells have 'little endogenous current', but could have easily determined this empirically using wildtype or non-transfected cells and the same recording conditions and APC platforms used in the present study. Both sets of additional cell-based experiments would have bolstered their silicone 'cell' dataset, removing two possible related explanations for the APC cell non-linearity (leftover hERG and/or endogenous conductances, both of which would be expected to be time-dependent and non-linear at positive voltages), and strengthening their main claim about a non-linear CaF leak effect without relying on a simple 'they look the same' argument. [...] Obviously there is a CaF-mediated non-linear biophysical phenomenon seen in the silicone-cell manual patch experiments, but acceptance of this manuscript and their (modified/clarified) claims about this affecting certain APC platform recordings requires actual cell-based data to compliment their historical APC datasets. [...] Also, the Conclusion stating that the CaF non-linear leak current is not due to endogenous (or non-blocked hERG) conductances is also not bourne out by the lack of manual patch clamp cell-based experimental data in this study, as outlined above.

Thank you for the suggestion. Additional experiments were carried out with non-transfected CHO cells using both a manual patch clamp system and the automated patch clamp platform with the same Ca-F containing solution, which are shown as new panels in Figure 2C and D.

The same nonlinear time-dependent current was observed even with non-transfected CHO cells, on both the automated and manual set ups, which confirms that the observed nonlinear current was not due to (potentially) remaining hERG conductance in Figure 2A and B.

As an APC user myself and a decades long patch clamper, I also have difficulty accepting the implication that the use of high internal fluoride and even low mM external divalents is a 'trick' solely employed for gigaseal APC recordings. I have lost track of the number of peer reviewed publications on voltage-gated Nav channels from leading academic groups, for example, that use this exact same recipe for their manual patch recordings, largely to ensure high resistance high fidelity recordings. Fig. 1A ignores this well-known tradition, assigning manual patch gigaseal resistances to the pipette glass-membrane tight seal alone. Thus, I would expect that a similar non-linear leak phenomenon would also be observable in many typical manual patch clamp recordings, but the authors notably did not run this experiment, and instead opted for a cell-free silicone-based biophysical manual patch pipette test. Thus, a claim or suggestion for 'correction' or close inspection of

APC recordings employing a CaF effect would thus equally apply to a great number of past and future manual patch clamp datasets, but the authors do not include this possibility in their Abstract or Conclusions.

As above, we have reworded the text to state that it is a problem with CaF_2 but not automated patch clamp platforms themselves, and to highlight that manual experiments with the same solutions are subject to the same problems (as indeed our new manual experiments show). We agree that there are manual patch experiments in the literature that used Ca/F solutions. We have further added the following references in Introduction to emphasise this, reads as:
"*For instance, a F− containing internal solution together with a Ca^{2+} containing external solution has been used with an early planar microfluidics setup (Kostyuk et al., 1975) and manual patching (Maltsev et al., 1997; Rugiero et al., 2003; Wang et al., 1996; Wendt et al., 1992), [...]*"
We have also further noted in Dicussion that some manual patch studies may need to be re-examined because of this nonlinear time-dependent leak, reads as:
"Some manual patch clamp findings in the literature may also need to be re-examined in light of these observations."

Also, their Discussion suggestions at the bottom on p7 are somewhat out-of-date, as many of these options have already been explored by experienced APC users (e.g. reducing Fl and Ca concentrations, use of alternate cations).

We could not find any reports of these currents in the literature, and therefore also haven't seen any reports on attempts to minimise them. But we would be happy to reference any examples that you can point us to.

Finally, the language in the Conclusions needs to be re-worded to limit their claims to a certain type of APC platform (i.e. remove the plural to 'APC platforms').

As above, we have reworded the Conclusions to state that the observation of nonlinear leak is due to the use of Ca-F containing solutions rather than APC. It now reads as:
"*We recorded a nonlinear and time-dependent current in the presence of Ca^{2+} and F− in patch clamp solutions (intended to form a CaF2 seal enhancer).*"

Competing Interests: N/A

Reviewer Report 14 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39339

?

Andrew Glazer

Vanderbilt Center for Arrhythmia Research and Therapeutics, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

Lei *et al.* describe a non-linear leak current that appears in the presence of fluoride, an ion used in automated patch clamp instruments to enhance seals. This unexpected leak current, not modeled by traditional leak current linear adjustment calculations, could interfere with the accurate characterization of ion channel properties on these instruments. Using manual patch clamp experiments with a silicone model of a cell, they reproduce the “extra” leak current and demonstrate that it disappears when the fluoride is washed out.

The paper is clear and well-written, and describes an important finding that has implications for the growing field of automated patch clamp electrophysiology. The authors make the raw data and code available, and some of their observations were reproduced by 2 different laboratories/instruments.

Major comments: The authors propose one solution to this problem, which is to do a full blocker-subtraction for all measurements, which could indeed help remove this “extra” leak current. However as the authors note, the properties of the seal might change over the course of the blocker addition (and some protocols are even longer to carry out than their staircase protocol). But what about actually removing the current with an internal solution exchange - another option the authors briefly mention in the discussion? Could they generate the initial tight seal on the SyncroPatch using an fluoride-containing internal solution and calcium-containing external solution, then do an internal solution exchange that removes the fluoride? Would the seals remain strong and the extra leak current go away, as they saw with Measurement 3 in manual patch clamp? If successful, this could demonstrate a relatively easy solution to this problem on the SyncroPatch. The authors briefly propose this experiment in the discussion as a future direction but it would be a nice addition to this paper to demonstrate it.

Please present multiple replicate cells for the I-V curves and show means and standard errors.

Minor comments:
The staircase protocol is a nice alternative to the standard hERG protocols and is described in the methods. But it would be helpful to mention it briefly in the Introduction and give a citation to the papers by the same authors that developed it to avoid confusing readers who might be expecting standard hERG protocols.

The paper is best read linearly including fully reading the Methods to understand the logic of the silicone and the experimental measurements. However I worry readers might skip the methods and jump to the results where there is not much explanation/motivation for the 3 measurements. This could be solved by adding more motivating text to the start of the results and to the start of each experimental measurement paragraph. For example, the first two paragraph of the methods could be moved to become the first two paragraphs of the results. And a sentence or two could be added to the start of each measurement paragraph in the results to describe the aim/goal of the measurement, for readers who didn’t read or skimmed the Methods.

Comparing 3-II to 3-III, although the leak current now appears to be “normal” (extra leak current removed), is the magnitude of the leak current higher after the fluoride solution is washed out? Could the authors present data on the resistances/leak current magnitudes before and after

fluoride washout? If there is a less tight seal, would this prevent the success of the internal solution exchange strategy on the SyncroPatch because the seals would decrease following washout?

After some confusion, I realized that X's in the the I-V curves in figures 2+3 each show 1 cell from the staircase protocol, with multiple X's at the same voltage. Please clarify this in the legend.

The author's charged plug model for the leak current is a bit speculative - but it is caveated as a "tentative" model/hypothesis.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Automated patch clamping.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 26 Oct 2021

Gary Mirams, University of Nottingham, Nottingham, UK

We thank the reviewer for their thoughtful review. This post relates to the changes introduced in Version 2 of the manuscript. The reviewer's comments are in bold and italic with our point-by-point responses below.

Major comments:
The authors propose one solution to this problem, which is to do a full blocker-subtraction for all measurements, which could indeed help remove this "extra" leak current. However

as the authors note, the properties of the seal might change over the course of the blocker addition (and some protocols are even longer to carry out than their staircase protocol). But what about actually removing the current with an internal solution exchange - another option the authors briefly mention in the discussion? Could they generate the initial tight seal on the SyncroPatch using an fluoride-containing internal solution and calcium-containing external solution, then do an internal solution exchange that removes the fluoride? Would the seals remain strong and the extra leak current go away, as they saw with Measurement 3 in manual patch clamp? If successful, this could demonstrate a relatively easy solution to this problem on the SyncroPatch. The authors briefly propose this experiment in the discussion as a future direction but it would be a nice addition to this paper to demonstrate it.

Thank you for the suggestion. Measurement III indeed suggested a potential method for removing the nonlinear time-dependent leak current. In this short 'Research Note' we are just hoping to draw attention to the problem rather than propose a particular solution - which may depend on the precise solutions being used, the cell types and the particular manual or automated patch platforms that are in use.

Please present multiple replicate cells for the I-V curves and show means and standard errors.

Thank you for the suggestion, more repeats of the experiments are shown in all figures for the I-V curves.

Minor comments:
The staircase protocol is a nice alternative to the standard hERG protocols and is described in the methods. But it would be helpful to mention it briefly in the Introduction and give a citation to the papers by the same authors that developed it to avoid confusing readers who might be expecting standard hERG protocols.

Thanks for the suggestion, it is now introduced in Introduction: "*The measurements were done under a voltage-clamp protocol used in Lei et al. (2019a), known as the "staircase protocol" (see top panel of Figure 2).*"

The paper is best read linearly including fully reading the Methods to understand the logic of the silicone and the experimental measurements. However I worry readers might skip the methods and jump to the results where there is not much explanation/motivation for the 3 measurements. This could be solved by adding more motivating text to the start of the results and to the start of each experimental measurement paragraph. For example, the first two paragraph of the methods could be moved to become the first two paragraphs of the results. And a sentence or two could be added to the start of each measurement paragraph in the results to describe the aim/goal of the measurement, for readers who didn't read or skimmed the Methods.

Thanks for the suggestion, indeed the paper was intended to be read linearly, as we wanted to give the logical flow of our observations and followed up experiments. We have now added more motivating text to the start of the results and to the start of each experimental

measurement paragraph in the Results section.

Comparing 3-II to 3-III, although the leak current now appears to be "normal" (extra leak current removed), is the magnitude of the leak current higher after the fluoride solution is washed out? Could the authors present data on the resistances/leak current magnitudes before and after fluoride washout? If there is a less tight seal, would this prevent the success of the internal solution exchange strategy on the SyncroPatch because the seals would decrease following washout?

The reviewer is correct, here the lower resistance suggests a larger leak which may be going 'around' rather than 'through' the crystal. We have now repeated the experiments (Measurements II and III), out of the four repeats, only two of them stayed approximately 'the same size' after the fluoride solution was washed out (as shown in the new Figure 4). We expect that there is still a potential to use the internal solution exchange strategy on the SyncroPatch but it would require further exploration and testing, hence we leave this as a potential future study.

After some confusion, I realized that X's in the I-V curves in figures 2+3 each show 1 cell from the staircase protocol, with multiple X's at the same voltage. Please clarify this in the legend.

New experiments for multiple cells/measurements have now been repeated and included in the I-V curves in all figures. We also explain the I-V curves contain more than one data point per cell at the same voltage in the figure caption, reads as
"*Note that each boxplot can contain more than one data point per cell due to the repeat of the voltage step in the protocol.*"

The author's charged plug model for the leak current is a bit speculative - but it is caveated as a "tentative" model/hypothesis.
Thank you for the comment, indeed we do not claim it was the conclusion drawn from the data presented in this manuscript. It was just a hypothesis that we made after observing the phenomenon.

Competing Interests: N/A

Reviewer Report 13 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39341

?

Gildas Loussouarn

CNRS, INSERM,the thorax institute, University of Nantes, Nantes, France

In the present manuscript, Lei and collaborators report the observation of a rectifying and time-dependent current, after the generation of Giga-seals enhanced by CaF crystals, a procedure that is unavoidable for several automated patch-clamp platforms using the planar patch configuration. This observation is of interest since such current, if not removed, may pollute the current under study.

The authors replaced a living cell by a drop of Sylgard in a conventional patch-clamp set-up to mimic an imperfect seal. Using this original procedure, they could observe a CaF-induced leak current, in absence of any plasma membrane endogenous current. However, some complementary experiments should be added to give more insights on the CaF-induced current and avoid any over-interpretation of the data.

1. The panels (A) and (B) of Figure 2 show strikingly similar recordings from two different laboratories using the same solutions in the same type of Syncropatch machine. Average of currents, at least for the I/V curve would be more convincing than comparing two representative cells.
2. Panel (C) is quite different to panel (A) and (B), with similar time dependent currents generated by a large depolarization (-80 to +40 mV) but not by smaller depolarization (0 to +40 mV). In Syncropatch systems, to avoid over–compensation and current oscillations that can disrupt the seal, Rseries feedback compensation of the recorded current is applied with a slow time constant. Given the high current amplitude (several nA), is it possible that the time-dependent current observed in panel C is due to slowly developing feedback compensation?

3. Figure 3, when switching internal and external solutions, it would be more relevant to also invert the polarity of the voltage protocol and to directly compare the superimposed currents. Moreover, as in point 1, average of currents, at least for the I/V curve would be more convincing.

4. Experiments in Figure 2 and 3 are quite different, with, in Figure 2, an automated patch-clamp system and a real cell whereas in Figure 3, a conventional patch-clamp system and an artificial cell. There may some other models without endogenous current, that can be used in automated patch-clamp, such as giant unilamellar vesicles[1]. Another option would be to test if the currents observed in Figure 2 and 3 are of the same nature, by replacing internal K^+ and/or Na^+ by the organic cation N-methyl-D-glucamine (NMDG) in the intracellular medium (be very careful if you need to use HF to prepare this solution, HF is a very corrosive and extremely toxic acid) or by replacing Cl^- by gluconate in the extracellular medium. Indeed, since the time-dependent current is an outward current, it may be carried by cations diffusing from the intracellular medium to the extracellular medium and/or by anions diffusing from the extracellular medium to the intracellular medium. Fluoride diffusion is unlikely the basis of the observed current, as suggested in the discussion: "*Furthermore, F^- has a higher mobility than Ca^{2+}, so F^- may preferentially move out through the imperfect seal and form crystals with Ca^{2+} on the Ca^{2+}-side of the membrane. This hypothesis is consistent with the direction of the 'extra' current in the leak in both Measurements I and II.*"

Minor points

1. In 'Data Analysis', "*E_{leak} is directly calculated from equation (1) using one of the voltage step*": it would be interesting to indicate if E_{leak} calculations gives values close to zero.

2. In the results, sentence "*The measured leak current was time-dependent when it was held at a constant voltage, and it showed a noticeable outward (positive) time-dependent current during an increase of voltage from zero to 40 mV*". I don't understand the difference between the two described time-dependence. I think the sentence could be simpler.

3. In the discussion: it is proposed that when fluoride is removed, CaF crystals remain, suggesting that both CaF and F^- need to be present to observe the time dependent and rectifying current. The fact that seals quality deteriorates upon F^- removal suggest that CaF crystals disappear, so the loss of the outwardly rectifying current may be due to the loss of crystals, and for instance, the loss of a rectifying current carried by intracellular cations in the crystal lattice, as suggested in "major point 4". There is no strong argument for the direct presence of fluoride as a cause of the outwardly rectifying current.

4. Authors should indicate other issues regarding the use of intracellular fluoride and extracellular calcium as a seal enhancer. Fluoride is (i) a phosphatase inhibitor and, obviously here, (ii) a calcium chelator preventing the use of calcium containing intracellular solutions. Using extracellular calcium at higher concentration than the physiological range alters channel gating[2], at least partly due to membrane charge screening[3].

References

1. Moparthi L, Zygmunt P: Human TRPA1 is an inherently mechanosensitive bilayer-gated ion channel. *bioRxiv*. 2020. Publisher Full Text
2. Ho WK, Kim I, Lee CO, Earm YE: Voltage-dependent blockade of HERG channels expressed in Xenopus oocytes by external Ca2+ and Mg2+.*J Physiol*. 1998; **507 (Pt 3)**: 631-8 PubMed Abstract | Publisher Full Text
3. McLaughlin SG, Szabo G, Eisenman G: Divalent ions and the surface potential of charged phospholipid membranes.*J Gen Physiol*. 1971; **58** (6): 667-87 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electrophysiology, patch-clamp.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 26 Oct 2021

Gary Mirams, University of Nottingham, Nottingham, UK

We thank the reviewer for the useful and insightful comments. Bold and italic below are the reviewer's comments with our point-by-point response below to describe updates in the revised version of the manuscript.

> The panels (A) and (B) of Figure 2 show strikingly similar recordings from two different laboratories using the same solutions in the same type of Syncropatch machine. Average of currents, at least for the I/V curve would be more convincing than comparing two representative cells.

Thanks for the suggestion. More repeats of the experiments were performed and included, and boxplots of the resulting I-V curves are shown in Figure 2. Further analysis of the time constants of the currents is now also included in Figure 3B.

> Panel (C) is quite different to panel (A) and (B), with similar time dependent currents generated by a large depolarization (-80 to +40 mV) but not by smaller depolarization (0 to +40 mV). In Syncropatch systems, to avoid over–compensation and current oscillations that can disrupt the seal, Rseries feedback compensation of the recorded current is applied with a slow time constant. Given the high current amplitude (several nA), is it possible that the time-dependent current observed in panel C is due to slowly developing feedback compensation?

Indeed the compensation may affect the shapes of the observed current, but since it couldn't estimate the membrane capacitance (as there was no cell), it would not be able to perform Rseries compensation. This has now been explained in the main text. Furthermore, we have conducted additional experiments with non-transfected CHO cells, and repeated these using both the conventional patch-clamp system and the automated patch-clamp system with the Ca-F containing solutions, with results shown in the new Figure 2C and D panels. We observed the same type of nonlinear current as those observed in Figure 2A and B and Measurement I.

> Figure 3, when switching internal and external solutions, it would be more relevant to also invert the polarity of the voltage protocol and to directly compare the superimposed currents.

We thank the reviewer for the suggestion. However, since we do not know exactly the electrochemical properties of the nonlinear time-dependent part of the current, for example

its reversal potential etc., we do not attempt to invert the polarity of the voltage protocol as it may not give an easily interpretable direct comparison.

> Moreover, as in point 1, average of currents, at least for the I/V curve would be more convincing.

More repeats of the experiments were undertaken and included for the I-V curves shown as boxplots.

> Experiments in Figure 2 and 3 are quite different, with, in Figure 2, an automated patch-clamp system and a real cell whereas in Figure 3, a conventional patch-clamp system and an artificial cell. There may some other models without endogenous current, that can be used in automated patch-clamp, such as giant unilamellar vesicles. Another option would be to test if the currents observed in Figure 2 and 3 are of the same nature, by replacing internal K+ and/or Na+ by the organic cation N-methyl-D-glucamine (NMDG) in the intracellular medium (be very careful if you need to use HF to prepare this solution, HF is a very corrosive and extremely toxic acid) or by replacing Cl- by gluconate in the extracellular medium.

We have included extra experiments with non-transfected CHO cells repeated using both the conventional patch-clamp system and the automated patch-clamp system with the Ca-F containing solutions. We observed the same type of nonlinear current as those observed in Figure 2A and B and Measurement I. We further analyzed the time constant of the observed current, as shown in new Figure 3. In particular Figure 3B shows that all the time dependent part of the observed current in all experiments A-E and Measurement I are the same.

> Indeed, since the time-dependent current is an outward current, it may be carried by cations diffusing from the intracellular medium to the extracellular medium and/or by anions diffusing from the extracellular medium to the intracellular medium. Fluoride diffusion is unlikely the basis of the observed current, as suggested in the discussion: "Furthermore, F— has a higher mobility than Ca2+, so F— may preferentially move out through the imperfect seal and form crystals with Ca2+ on the Ca2+-side of the membrane. This hypothesis is consistent with the direction of the 'extra' current in the leak in both Measurements I and II."

Thank you for this comment, you are correct that the nonlinear outward current would be either cations moving from the intracellular medium to the extracellular medium and/or anions moving from the extracellular to the intracellular. The quoted text from the main text "*Furthermore, F- has a higher mobility [...]*" simply refers to where the crystal would form, which would happen before the measurements were done, hence we did not mean to suggest that the observed current *was* fluoride moving from the intracellular medium to the extracellular medium, just that it was caused by it. We agree that the text was confusing, we've replaced it with:
"*Our leading conjecture is that the nonlinear time-dependent leak current is ordinary linear leak through imperfect seal with a conductance that changes nonlinearly and time-dependently with voltage. We speculate that the reason for this 'extra' current could be defects in the CaF2 crystals: F—* has a higher mobility than Ca2+, so F— may preferentially move back into the cell from

imperfections in the crystal when voltage is high. The extra defects in the crystal that result (sites missing fluoride, Huisinga, 1999) could allow a larger non-selective leak, forming the measured current. This process would reverse when voltage is low, with fluoride returning to 'plug the holes' in the crystal and reducing leak conductance, giving the observed asymmetry in leak current. This is a similar concept to e.g. positively charged polyamines causing block of inward rectifier potassium channels at positive potentials (Fakler et al., 1995). That is, neither fluoride nor the polyamines contribute strongly to a measured current but are the cause of its block. This hypothesis is consistent with the direction of the 'extra' current in the leak in both Measurements I and II."

> Minor points
In 'Data Analysis', "Eleak is directly calculated from equation (1) using one of the voltage step": it would be interesting to indicate if Eleak calculations gives values close to zero.

The estimated Eleak values across all recordings are 8.14 ± 3.38 mV (mean ± SEM) which is relatively close to zero, but we do observe a fair amount of variation in Eleak. We have included this in the main text.

> In the results, sentence "The measured leak current was time-dependent when it was held at a constant voltage, and it showed a noticeable outward (positive) time-dependent current during an increase of voltage from zero to 40 mV". I don't understand the difference between the two described time-dependence. I think the sentence could be simpler.

We have reworded the sentence, which reads as:
"*The measured leak current was time-dependent when it was held at a constant voltage, this is most noticeable for the outward (positive) current during an increase of voltage from 0 to +40 mV.*"

> In the discussion: it is proposed that when fluoride is removed, CaF crystals remain, suggesting that both CaF and F- need to be present to observe the time dependent and rectifying current. The fact that seals quality deteriorates upon F- removal suggest that CaF crystals disappear, so the loss of the outwardly rectifying current may be due to the loss of crystals, and for instance, the loss of a rectifying current carried by intracellular cations in the crystal lattice, as suggested in "major point 4". There is no strong argument for the direct presence of fluoride as a cause of the outwardly rectifying current.

The reviewer is correct that in the previous Measurement III, the lower resistance suggests a larger leak which may be going 'around' rather than 'through' the crystal or even be due to the loss of the crystal. Additional experiments were performed for Measurements II and III. In two of the four repeats, the seal quality (the leak current size) stayed the same whilst the nonlinear time-dependent current still disappeared after the fluoride solution was washed out. This is shown in Figure 4 (a new version of Figure 3), supporting the proposed hypothesis.

> Authors should indicate other issues regarding the use of intracellular fluoride and extracellular calcium as a seal enhancer. Fluoride is (i) a phosphatase inhibitor and,

obviously here, (ii) a calcium chelator preventing the use of calcium containing intracellular solutions. Using extracellular calcium at higher concentration than the physiological range alters channel gating, at least partly due to membrane charge screening.

Thank you for the suggestion. The following text has now been added to the end of Discussion:
"*In addition to the extra nonlinear time-dependent leak current, the use of intracellular fluoride and extracellular calcium as a seal enhancer could also give rise to other problems. Using intracellular fluoride, as a calcium chelator, prevents the use of calcium containing intracellular solutions, and fluoride is also a phosphatase inhibitor (Guranowski 1990). Using extracellular calcium at higher concentrations than the physiological range can also alter channel gating (Ho et al., 1998), at least partly due to membrane charge screening (McLaughlin et al., 1971).*"

Guranowski A. Fluoride is a strong and specific inhibitor of (asymmetrical) Ap4A hydrolases. *FEBS letters*. 1990; 262(2):205-8.

Ho WK, Kim I, Lee CO, Earm YE. Voltage-dependent blockade of HERG channels expressed in Xenopus oocytes by external Ca2+ and Mg2+. *J Physiol.* 1998; 507 (Pt 3): 631-8

McLaughlin SG, Szabo G, Eisenman G. Divalent ions and the surface potential of charged phospholipid membranes. *J Gen Physiol*. 1971; 58 (6): 667-87

Competing Interests: N/A